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Characteristics of a Newly Isolated Fungus, *Geotrichum candidum* Dec 1, Which Decolorizes Various Dyes

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A fungus, *Geotrichum candidum* Dec 1, newly isolated from soil as a dye-decolorizing microorganism, decolorized 18 kinds of reactive, acidic and dispersive dyes and 3 model compounds on a solid medium, showing a broad spectrum of decolorization. Except for dispersive dyes, all the dyes used on the solid medium were also decolorized even in a liquid medium, although the decolorizing rates varied depending on the dye structure. By repeated addition of one dye, Reactive blue 5, about 12 g/l of the dye was degraded without significant decline of activity, showing the resistant property of Dec 1 to a high concentration of the dye. An energy source and oxygen were essential for the expression of decolorizing activity; the optimal temperature was 30°C. A crude extracellular enzyme solution, in which the decolorizing activity was more than 100 times that of the Dec 1 culture broth, showed peroxidase activity, indicating that some peroxidases are responsible for dye-decolorization.

[Key words: decolorization, dye, *Geotrichum candidum*, peroxidase]

Synthetic dyes are released into the environment in effluents arising from major processes in the textile and dyestuff industries. As they are relatively recalcitrant to biodegradation, the elimination of colored effluents in wastewater treatment system is mainly based on physical or chemical procedures such as adsorption, concentration, chemical transformation and incineration. Although these methods are effective, they suffer from such shortcomings as high cost, formation of hazardous by-products and intensive energy requirements. Therefore, as a better alternative, microbial biodegradation methods are receiving attention. Several strains of dye- or colored material-degrading microorganisms have been reported. The best studied fungus is the white-rot fungus *Phanerochaete chrysosporium* which degrades lignin by producing lignin peroxidase, and details of its degradation mechanism have been elucidated (1–6). *Pleurotus ostreatus* (7), *Coriolus versicolor* (8, 9), and *Streptomyces* spp. (5, 6, 10) are among other microorganisms that have also been investigated as decolorizing microbe candidate. However, the number of the microorganisms that have been studied for decolorization of dyes is limited. The effectiveness of microbial treatment depends on the survival, adaptability and activity of the selected microorganism. Here, we screened for microorganisms which showed a rather wide spectrum of degradability against several dyes, and a new fungus which exhibited decolorizing ability towards various dyes was isolated.

MATERIALS AND METHODS

Screening of decolorizing microorganisms Dye-decolorizing microorganisms were isolated from several soil samples by spreading soil suspensions in water onto GPY agar or PDA medium containing three kinds of dyes at a 200 ppm each. GPY agar medium contains (g/l) glucose 10, Polypepton 3, yeast extract 7, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$, and agar 15 (pH=7). PDA medium (Eiken Chemical Co., Tokyo) consists of (g/l) potato extract 200, glucose 20, and agar 15 (pH=5.6). The dyes used for screening were Color Index (C.I.) Reactive blue 114 which is representative of reactive dyes that contain an anthraquinone group as the chromophore; C.I. Acid blue 324, one of acidic dyes containing an azo group as the chromophore; and C.I. Dispersive blue 79, one of dispersive dyes with an azo chromophore. Agar plates spread with a soil suspension were incubated at 30°C for 3 weeks. Colonies which formed transparent dye zones were re-suspended in GPY solution and purified. The purified colonies were subjected to the same procedure described above and the decolorizing ability of each purified microorganism was confirmed.

Dyes used for experiments The dyes used are listed in Table 2. Besides the dyes, chemicals with simplified structures of C.I. Reactive blue 5, named AQ-1,1-amino-4-(3-amino-4-sodium sulfonoanilino)-2-sodium anthraquinone sulfonate, and AQ-2, 1-amino-4-methylamino-2-sodium anthraquinone sulfonate and of C.I. Reactive red 33, named as AZ-1,1-hydroxy-6-methylamino-3-sodium naphthalene sulfonate-2-azo-(4′methoxy-2′sodium benzene sulfonate) were also used. The structures of four dyes and of these chemicals are shown in Fig. 1.

Decolorizing test using the purified fungus Dec 1 on solid medium Each dye, which was membrane-filtered with a 0.45 μm cellulose nitrate filter (Advantec, Tokyo), was mixed with PDA medium to give a final concentrations of 100–200 ppm on agar plates. The fungal mycelia were transferred to the center of each plate by a sterile platinum ring. The plates were then incubated at 28°C and their surface appearance was visually observed daily.

Decolorizing test using the purified fungus Dec 1 in liquid medium All the mycelia of the purified fungus grown on PDA medium for 6 d were suspended in sterile distilled water. After filtering through gauze to remove fungal mycelia, a spore suspension of about 10^7 colony forming unit (cfu)/ml was prepared. Five ml of this suspension was added to 150 ml potato dextrose (PD) medium (pH=6) in 500-ml conical flasks and shaken at 120 strokes per minute (spm) at 30°C for 6 d.

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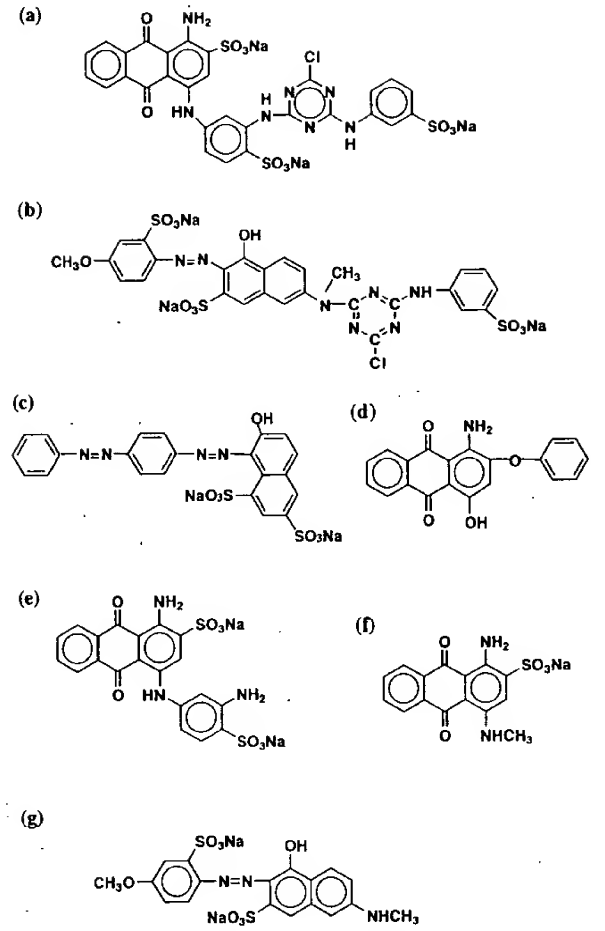

FIG. 1. Structure of selected dyes and the model chemicals used. (a) Color Index (C.I.) Reactive blue 5; (b) C.I. Reactive red 331; (c) C.I. Acid red 73; (d) C.I. Dispersive red 60; (e) AQ-1; (f) AQ-2; (g) AZ-1.

A water solution of each dye filtered with a membrane filter was added into each flask to give a concentration of 100–200 ppm. Periodically, 0.1–1.0 ml of the culture broth was sampled in an Eppendorf tube and centrifuged at 12,000 rpm for 3 min to obtain the supernatant. The dye disappearance of each supernatant was determined spectrophotometrically by monitoring the absorbance at the wavelength maximum for each dye. The decolorizing rate of each dye by cell-free culture was determined by the difference in the absorbance at the maximum wavelength between the initial and sampling values at each sampling time. A calibration curve of the relationship between absorbance and dye concentration as prepared beforehand, and the decolorizing rate given by the absorbance difference was converted to a concentration difference for each dye per unit time. The maximum absorbance wavelength of each dye (Table 3) was determined beforehand by scanning with a spectrophotometer (UV-240, Shimadzu, Kyoto).

Preparation of extracellular or intracellular crude enzyme solutions of Dec 1 After fungal cells of Dec 1 were grown for 3 d in flasks containing 100 ml PD medium, 50 ppm of C.I. Reactive blue 5 dye was added and mixed and its complete decolorization within 12 h was confirmed. The cell suspension was then centrifuged at 8,000 rpm for 20 min and the supernatant was gently mixed with ammonium sulfate powders to a level of 80% and the proteins were then precipitated by mild mixing. After standing for 1 h, the precipitate was collected by centrifugation at 12,000 rpm for 10 min. The sediment was then solubilized with 25 mM citric acid buffer (pH = 5) and the solution was dialyzed against the citric acid buffer to remove ammonium sulfate. The dialyzed solution was filtered with a cellulose nitrate filter (0.45 μm) and preserved at a 4°C room as an extracellular crude enzyme solution. These procedures were conducted at a 4°C-room.

The fungal cell mass (about 300 mg dry weight) collected by centrifugation was mixed with quartz sand in a mortar and disrupted by hand at 4°C. Twenty-five ml of citric acid buffer (pH = 5) was added and the mixture was centrifuged at 12,000 rpm for 10 min. The supernatant collected was used as a cell-free, intracellular crude enzyme solution.

TABLE 1. Characteristics of a newly isolated fungus Dec 1

Colonies on potato dextrose agar (PDA) medium grow rapidly with a diameter of about 75 mm in 5 d at 25°C.
Colony surfaces are floccose to cottony and white in color. Exudate is not present.
Colonies on malt extract agar medium are similar to those on PDA.
Colonies on corn meal agar medium are 38–51 mm, effuse and spreading, and hyaline to subhyaline in color.
Optimum pH in Czapek-yeast extract medium: 6 at 25°C.
Optimum temperature on PDA medium: 25°C.
Mycelia, 1.6–4.8 μm in width, are hyaline, septate, more or less specialized into broad, radiating vegetative hyphae which branch dichotomously, and narrow, lateral, sporulating hyphae which also branch.
Conidia are arthrospore-type, formed in chains by separation and breaking of the sporulating hyphae, and sometimes intercalary in the broad vegetative hyphae as well.
Conidia chains are aerial, erect or decumbent, or flat. Conidia are cylindrical at first, later varying from barrel-shape to ellipsoidal, oblong, subglobose and 2.4–4.8 μm × 2.4–16 μm.

RESULTS

Isolation of decolorizing fungus Several strains of fungi and bacteria which formed clear halos on the dye plates were isolated as dye-decolorizing microorganisms. Among these, one fungal strain that showed high dye-degradation ability and a broad dye-degrading spectrum was selected for further experiments. The fungus was identified as *Geotrichum candidum* Dec 1, based on its characteristics as shown in Table 1 and References 11,12. On the solid PDA medium, the fungus degraded 18 kinds of dyes during 4-d incubation: 15 reactive, 2 acidic and 1 dispersive as well as the three model compounds (Table 2). The dyes in Table 2 that were less degraded over 4 d were eventually decolorized completely by further incubation up to 7 d. However, Dispersive blue 79 was hardly degraded on solid medium in 4 d, and the degree of decolorization remained unchanged even after 2 weeks, while Dispersive red 60 changed its original color to pale yellow after 2 weeks. With the exception of the dispersive dyes, all the dyes that were decolorized on the solid medium were also degraded in liquid PD medium, although the decolorizing

TABLE 2. Degree of decolorization of various dyes by *G. candidum* Dec 1 on PDA medium after 4-d incubation at 28°C

Color Index	Chromophore	Concentration added (ppm)	Decolorized diameter (cm)[a]	Relative value[b]
Reactive blue 5	Anthraquinone	100	8.5	1.0
Reactive blue 19	Anthraquinone	100	8.0	0.94
Reactive blue 114	Anthraquinone	100	7.5	0.88
Reactive blue 182	Azo	100	8.0	0.94
Reactive black 5	Azo	100	8.0	0.94
Reactive red 33	Azo	100	7.0	0.82
Reactive red 120	Azo	100	5.5	0.65
Reactive red 123	Azo	100	6.5	0.77
Reactive red 187	Azo	100	4.0	0.47
Reactive red 202	Azo	100	6.5	0.77
Reactive red 225	Azo	100	6.0	0.71
Reactive orange 13	Azo	100	3.5	0.41
Reactive orange 30	Azo	100	4.0	0.47
Reactive violet 23	Azo	100	7.5	0.88
Reactive yellow 2	Azo	100	3.5	0.41
Acid red 73	Azo	100	6.5	0.77
Acid blue 324	Azo	100	7.0	0.82
Dispersive red 60	Anthraquinone	100	3.0	0.35
Dispersive blue 79	Azo	100	n.d.	n.d.
AQ-1[c]	Anthraquinone	100	7.5	0.88
AQ-2[c]	Anthraquinone	100	8.5	1.0
AZ-1[d]	Azo	100	6.0	0.71

[a] Decolorized diameter after 4-d incubation.
[b] Relative value of diameter to diameter of Reactive blue 5.
[c] Model chemicals of Reactive blue 5.
[d] Model chemical of Reactive red 33.
n.d., Not detected.

rate varied in each sample (Table 3). Photographs of the decolorized plates of four dyes on PDA medium after 2–5 d incubation as shown in Fig. 2, and the structure of each of these dyes is given in Fig. 1. In Czapek-Dox agar medium, which contains only glucose and mineral salts, decolorization of the dyes listed in Table 2 was complete although a period of 7–10 d was needed (data not shown).

Decolorization by strain Dec 1 in liquid medium

The time courses of growth and pH of Dec 1 in PD medium are shown in Fig. 3. The growth curve was obtained by sampling each flask containing 100 ml of PD

TABLE 3. Decolorizing rates of dyes in culture broth of *G. candidum* Dec 1 grown in PD liquid medium for 6 d and by extracellular crude enzyme solution prepared from the culture broth of Dec 1

Color Index	Max. absorbance wavelength (nm)	Concentration added (ppm)	Decolorizing rate (ppm/h)[a]	Decolorizing rate (ppm/h)[b]
Reactive blue 5	600	200[c] (100)[d]	95	2442
Reactive blue 19	590	150 (70)	42	1740
Reactive blue 114	620	200 (100)	71	702
Reactive blue 182	610	300 (120)	181	3018
Reactive black 5	598	60 (30)	11	20.4
Reactive red 33	500	100 (50)	22	31.4
Reactive red 120	535	100	13	n.d.
Reactive red 123	500	100	7.8	n.d.
Reactive red 187	533	100	3.3	n.d.
Reactive red 202	548	150	6.2	n.d.
Reactive red 225	530	80	5.0	n.d.
Reactive orange 13	490	100	2.1	n.d.
Reactive orange 30	400	250	2.5	n.d.
Reactive violet 23	560	200	15	n.d.
Reactive yellow 2	390	250 (100)	3.6	70.8
Acid red 73	507	40	2.9	n.d.
Acid blue 324	605	200	20	n.d.
AQ-1	600	200 (60)	46	366
AQ-2	635	160 (50)	40	1080

[a] Decolorizing rate by the culture broth of Dec 1.
[b] Decolorizing rate by crude enzyme solution containing about 0.5 μg protein/ml.
[c] Initial dye concentration for measuring activity by the culture broth of Dec 1.
[d] Initial dye concentration for measuring activity by the crude enzyme solution.
n.d., Not detected.

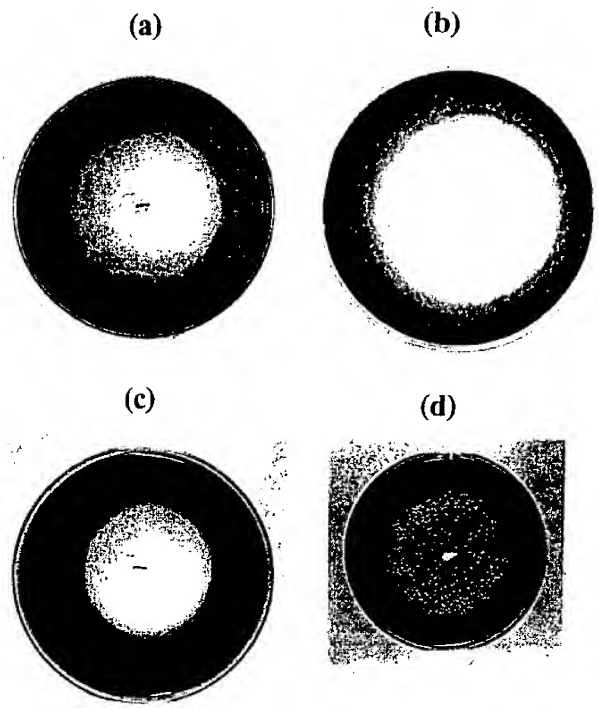

FIG. 2. Photographs of PDA medium containing the dyes in Fig. 1 decolorized by *G. candidum* Dec 1. (a) C.I. Reactive blue 5 after 2 d and 18 h incubation; (b) C.I. Reactive red 33 after 3 d and 5 h incubation; (c) C.I. Acid red 73 after 2 d and 18 h incubation; (d) C.I. Dispersive red 60 after 5 d incubation.

medium and drying the whole fungal cell mass in a flask to obtain each data point. The growth leveled off in 3 d, and a decline in the dry weight was observed, mainly due to autolysis of the cells. The pH declined from 6 to 4.5 in 3 d and maintained an almost constant level of around 5 thereafter for 2 weeks. Figure 4 shows the change in the decolorizing activity of Dec 1 toward C.I. Reactive blue 5 dye and the glucose concentration measured by the Glucose-B test (Wako Pure Chemicals Industries Ltd., Osaka). When the dye at about 200 ppm was

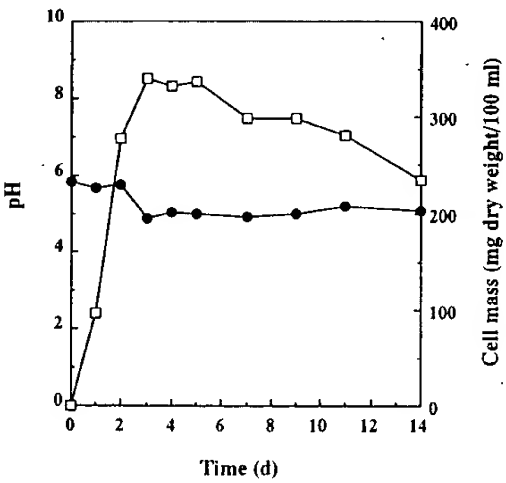

FIG. 3. Time courses of pH and growth expressed as dry weight when *G. candidum* Dec 1 was grown in liquid PD medium at 30°C.

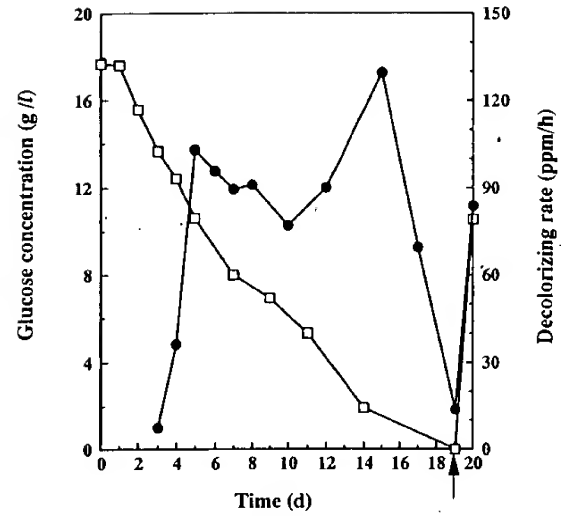

FIG. 4. Decolorizing activity of Reactive blue 5 dye by Dec 1 and glucose concentration in liquid PD medium at 30°C after the growth had leveled off. The black arrow indicates the time when 10 g/l of glucose was added.

added after 3 d, when the growth had leveled off and the absorbance of the cell-free culture was measured, the decolorizing activity appeared quickly, reached a maximum of 100 ppm/h in 5 d and then declined to 77 ppm/h in 10 d. However, the activity increased again to 130 ppm/h in 14 d. A decrease in glucose concentration occurred significantly after 3 d and lasted for 19 d until its exhaustion. When the glucose reached less than 2 g/l, the deterioration of the activity was significant. Since supplementation of 10 g/l glucose on day 19 (shown by a black arrow in the figure) led a quick recovery of the decolorizing activity, it is clear that glucose is essential as an energy sources to maintain the decolorizing activity.

Figure 5 presents the changes in the decolorization rate when the initial concentration of Reactive blue 5 dye was varied. At 400–1,600 ppm, the rate generally showed a steady increase. However, at 3,200 ppm, after slower degradation for 4 h, which was presumably due to the in-

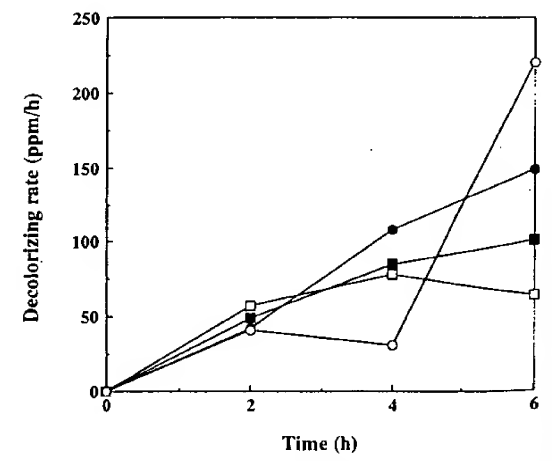

FIG. 5. Decolorizing rates at various initial dye concentrations of Reactive blue 5. □, 400 ppm; ■, 800 ppm; ●, 1,600 ppm; ○, 3,200 ppm.

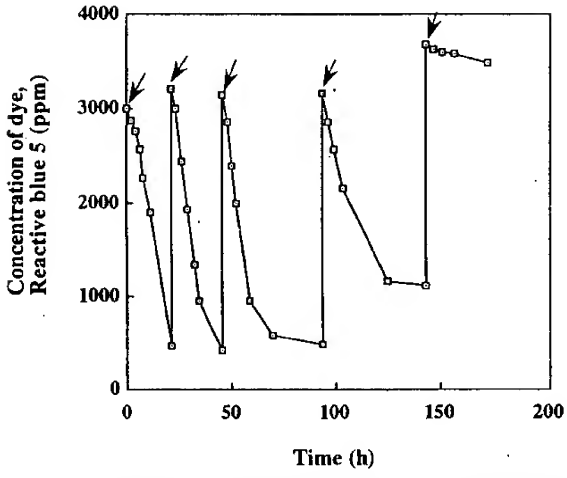

FIG. 6. Degradation of Reactive blue 5 dye by repeated addition (as shown by black arrows) of 3,000 ppm each time. This addition experiment was followed by the experiment in Fig. 5.

itial inhibition of the dye to the cell activity, enhancement of the rate was attained in 6 h. Therefore, about 3,000 ppm of the dye was added repeatedly (shown by black arrows in Fig. 6). About 12 g/l of the dye was totally degraded during this repeated addition without a significant decline in activity up to the fifth addition of the dye, after which the activity declined remarkably.

The effect of temperature on the decolorizing activity rate is shown in Fig. 7. The optimal temperature is seen to be 30°C. The cell mass of Dec 1 after 7 d at each temperature did not differ greatly except at 37°C.

The initial pH of PD medium was set at 4, 5, 6 and 7, but in each case, the pH value was around 5 after 5-d incubation. Since no significant difference in the decolorizing rate of cells collected in 5 d was seen (data not shown), the initial pH of PD medium was fixed at 6 in all other experiments.

O_2 requirement in decolorization Cell mass harvested by the procedure described above was grown in either aerobic or anaerobic conditions. N_2 gas was

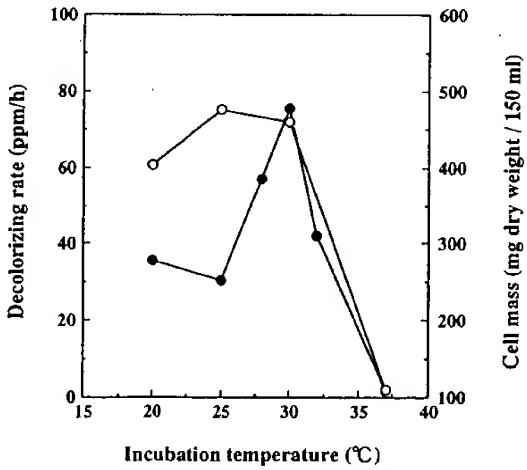

FIG. 7. Effect of temperature on the decolorizing rate (●) and cell mass (○).

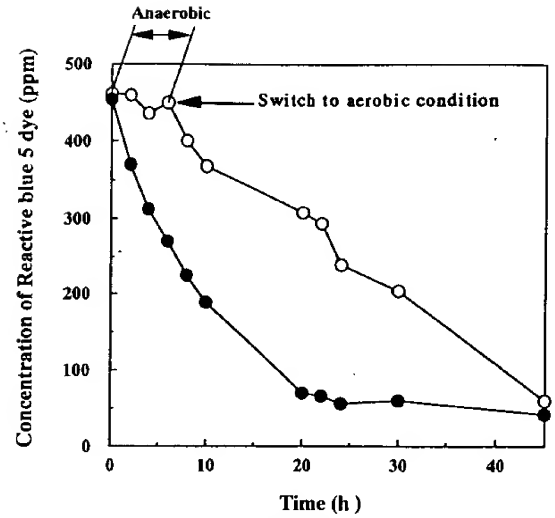

FIG. 8. Degradation of Reactive blue 5 dye under aerobic (●) and anaerobic (○) conditions by Dec 1.

flushed through a sterile filter into PD medium for 10 min to replace O_2 before inoculation of the fungal cell mass. Then, the cell mass and Reactive blue 5 dye at 500 ppm were mixed in a flask and the decolorizing activity was monitored (Fig. 8). It is clear that decolorizing activity was not expressed under the anaerobic condition, in sharp contrast to the activity under aerobic condition. When the supply of air was started after 8 h, expression of decolorizing ability was resumed, suggesting that oxygen is essential for dye-degradation by Dec 1.

Characteristics of crude enzyme solution responsible for decolorization The intracellular crude enzyme solution prepared by disruption of the fungal cells was mixed with 0.2 mM H_2O_2 and Reactive blue 5 dye at 50 ppm and incubated at 28°C with shaking at 120 spm. However, no dye-degrading activity was observed (data not shown). Figure 9 shows the decolorizing activity of the extracellular crude enzyme solution containing 66 μg protein/ml in a mixture of 0.2 mM H_2O_2 and 50 ppm

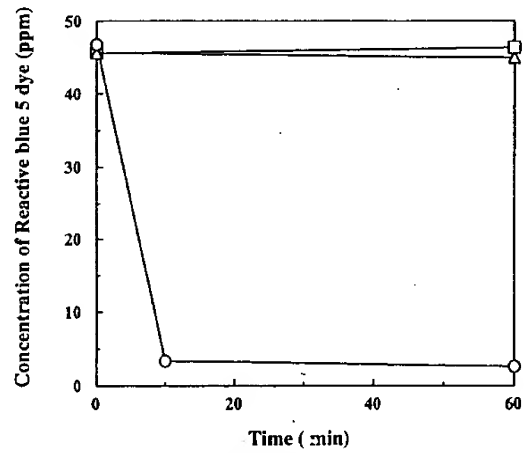

FIG. 9. Decolorizing activity of Reactive blue 5 dye by extracellular crude enzyme solution with H_2O_2 (○), crude enzyme solution without H_2O_2 (△) and heat-denatured enzyme solution with H_2O_2 (□).

Reactive blue 5 dye. The extracellular crude enzyme solution revealed peroxidase activity. No activity was seen when the dye was mixed with crude enzyme solution without H_2O_2, or with crude enzyme solution denatured at 85°C for 15 min plus H_2O_2. Table 3 shows the decolorizing rate of crude enzyme solution containing about 0.5 μg protein/ml.

DISCUSSION

To date, the decolorizing abilities of isolated microorganisms have been reported for limited numbers of dyes. These include *P. ostreatus* for one polymeric dye (7); *C. versicolor* for melanoidin (9); *Streptomycetes* for three anthron-dyes (10); *P. chrysosporium* for three polymeric dyes (1), three azo dyes (4) and four azo and heterocyclic dyes (2); and *Streptomyces* sp. and *P. chrysosporium* for several azo dyes and their simplified chemical substitutes (5, 6).

Here, we first demonstrated that a yeast-like fungus, *G. candidum* Dec 1, degrades a variety of dyes commercially available both in solid and liquid cultures (Tables 2 and 3) and that the rate of decolorization by *G. candidum* Dec 1 is dependent on the energy-source and oxygen. Although the fungus oxidized lignin-related compounds (12) or herbicides (13), suggesting the existence of peroxidase-like enzymes, we also showed that *G. candidum* has a broad decolorizing spectrum toward dyes, mainly by producing extracellular peroxidase-type enzymes. If microbial strains are specific to single dyes, they are not considered to be of any practical value for degrading the mixtures of dyes that occur in most wastewaters.

As shown in Fig. 6, a total of 12 g/*l* of dye was degraded without a significant decrease in the decolorizing rate with repeated addition of the dye. When 12 g/*l* of the dye was added initially to the PD medium, the fungus degraded almost all of the dye within 2 d, suggesting that the degradation activity of the fungus was rather resistant to a high dye concentration (data not shown). *G. candidum* occurs widely in soil and water, and excellent growth was observed over a pH range of 4–7 and in a temperature range of 20–30°C, as shown in Fig. 7. This fungus utilizes acetate, ethanol and acetone as energy source (15) and these less expensive carbon sources may be used instead of glucose in practical decolorization applications. These characteristics give the fungus the potential to remove colored materials not only in a reactor-type system but also in bioremediation in soil.

As Dec 1 decolorized the model compounds AQ-1 and AQ-2 and AZ-1, the fungus is believed to attack the chromophores directly. The effectiveness of decolorization depends on the structure and complexity of each dye, as the rate of decolorizing varied from one dye to another (Tables 2 and 3). In Table 3, a comparison of the decolorizing activity between the culture broth and the crude enzyme solution is made. The degradation rate for the enzyme solution is seen to be significantly enhanced more than 100-fold for some of the dyes. As the crude enzyme solution was prepared from the culture broth after 3-d cultivation when the growth of Dec 1 reached a stationary phase, and the enzyme activity lasted for a longer period of time, the peroxidase(s) produced by the fungus will be inducible. The dyes themselves are not inducers because the enzyme activity appeared in PD medium. Figure 4 shows two peaks of activity during cultivation period, which may indicate the production of different kinds of peroxidases, the involvement of enzymes other than peroxidases, or higher production of the same enzymes(s) at the later time. The purification and characterization of these enzymes is now on progress.

Enzymatic treatment to remove or decolorize aromatic chemicals (16, 17) is an alternative to the conventional methods. Lignin peroxidase has been shown to break nonspecifically many aromatic and substituted aromatic rings (2). Horseradish peroxidase (HRP) has been used in basic research in this area (18, 19), but other microbial peroxidases which have different properties than HRP are needed. The application of peroxidase may be one of available methods to decolorize colored materials. Lignin-degrading ability is considered to be related to dye-degrading ability (1). In our preliminary experiments, Dec 1 degraded veratryl alcohol, which is a model substrate, to give the activity of lignin peroxidase (data not shown). Dyes, thus provide a suitable means for the selection of microorganisms capable of degrading lignin, mainly because dye-degradation is visibly observable.

In our experiments, the rate of decolorization was slower in nitrogen-sufficient GPY medium, while the loss of color of dyes in nitrogen-insufficient PDA medium occurred quickly (data not shown). As Dec 1 showed dye-decolorizing ability even in Czapek-Dox medium, nitrogen-limitation is favorable for the fungal expression of genes responsible for the production of peroxidase. A similar phenomenon was reported with *P. chrysosporium* (2, 4), in that the lignin-degrading activity was much higher under a nitrogen-limited than a nitrogen-rich condition. The fact that no excessive nutrient is needed for the fungus will be advantageous for its practical application.

A more detailed understanding of dye-degradation by Dec 1 is required in order that fungus-based treatment systems for the cleanup of dye industry effluents and for bioremediation of dye-contaminated soil can be developed.

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Purification and Characterization of a Novel Peroxidase from *Geotrichum candidum* Dec 1 Involved in Decolorization of Dyes

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A peroxidase (DyP) involved in the decolorization of dyes and produced by the fungus strain *Geotrichum candidum* Dec 1 was purified. DyP, a glycoprotein, is glycosylated with *N*-acetylglucosamine and mannose (17%) and has a molecular mass of 60 kDa and an isoelectric point (pI) of 3.8. The absorption spectrum of DyP exhibited a Soret band at 406 nm corresponding to a hemoprotein, and its $Na_2S_2O_4$-reduced form revealed a peak at 556 nm that indicates the presence of a protoheme as its prosthetic group. Nine of the 21 types of dyes that were decolorized by Dec 1 cells were decolorized by DyP; in particular, anthraquinone dyes were highly decolorized. DyP also oxidized 2,6-dimethoxyphenol and guaiacol but not veratryl alcohol. The optimal temperature for DyP activity was 30°C, and DyP activity was stable even after incubation at 50°C for 11 h.

The discharge to the environment of 10 to 15% of the synthetic dyes produced (42) causes environmental problems. These dyes are poorly biodegradable because of their structures, and treatment of wastewater containing dyes usually involves physical and/or chemical methods. Although these treatment methods are efficient, they may result in the production of toxic by-products and/or require high levels of energy. Microbial decolorization has been proposed as a less expensive and less environmentally intrusive alternative. Various bacteria and fungi have decolorizing abilities, and an extensive review of microbiological decolorization has been made (3); in many cases adsorption of dyes to the microbial cell surface is the primary mechanism of decolorization (22).

Azo dyes may be microbially degraded under anaerobic (28, 52) or aerobic (8, 13, 30, 32, 42, 47) conditions or in aerobic and anaerobic two-stage systems (39). Enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, all of which are involved in lignin degradation, participate in the decolorization of the dyes (6–8, 13, 30, 47). Recently, another such peroxidase was purified from *Pleurotus ostreatus* that was found to be different from MnP, LiP, and horseradish peroxidase (HRP) (17, 41). However, few studies have been made of the enzymatic degradation of anthraquinone dyes, which are xenobiotic chemicals similar to azo dyes but different in structure (11, 22, 31, 47).

Previously, we reported that *Geotrichum candidum* Dec 1, a newly isolated decolorizing fungus, decolorized 21 types of reactive dyes, including azo and anthraquinone dyes (18). The broad decolorization spectrum of this strain suggested the involvement of extracellular peroxidase-type enzymes. Our objectives in this study were to purify and characterize the novel peroxidase (DyP) that is responsible for the dye-decolorizing activity of *G. candidum* Dec 1.

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MATERIALS AND METHODS

Organism and culture media. *Geotrichum candidum* Dec 1 was isolated from soil (18–20). Cells of Dec 1 from a potato dextrose agar (PDA) (Eiken Chemical Co. Ltd., Tokyo, Japan) slant stored at 4°C were transferred to fresh PDA plates and incubated at 30°C for 6 days; all of the mycelia on the PDA plates were suspended in sterile distilled water. After being filtered through gauze to remove fungal mycelia, a spore suspension of about 10^7 CFU/ml was prepared. Potato dextrose broth (Difco) was used for liquid cultivation.

Enzymes and chemicals. HRP (Wako Chemical Co. Ltd., Osaka, Japan) was used for comparison with the newly purified peroxidase involved in decolorization (DyP). The main dyes used were Reactive Blue 5 (RB5), an anthraquinone dye, and model compounds of RB5 (Nippon Kayaku Co., Ltd., Tokyo, Japan), i.e., 1,4-diamino-2-sodium anthraquinone sulfonate (AQ-2'), 1-amino-4-methylamino-2-sodium anthraquinone sulfonate (AQ-2), and 1-amino-4-(3-amino-4-sodium-sulfonoanilino)-2-sodium anthraquinone sulfonate (AQ-1). The other reagents used were of the highest quality available.

Enzyme purification. One hundred fifty milliliters of potato dextrose broth in a 500-ml flask was inoculated with 5 ml of spore suspension and shaken at 30°C at 120 strokes per min for 6 days. Unless otherwise stated, all procedures were performed at 4°C. The supernatant (4.4×10^3 ml) obtained by centrifugation of the culture broth at 7,200 × *g* for 20 min was passed through a glass fiber filter (GC 50; Toyo Roshi Co. Ltd., Tokyo, Japan) to remove polysaccharides produced during cultivation. The filtrate was concentrated to 60 ml by ultrafiltration with a YM 10 membrane (Amicon Grace Japan, Tokyo, Japan). The concentrate was dialyzed with 25 mM piperazine buffer with a counterion of piperazine chloride (pH 5.5) to 80 ml and then concentrated to 17 ml by ultrafiltration with Centriprep 10 (Amicon Grace Japan). The pooled fraction (17 ml) was loaded onto a Super Q 650M (Tosoh Co. Ltd., Tokyo, Japan) column (2.8 by 6.0 cm) previously equilibrated with the same buffer (pH 5.5). The column was subsequently washed with 200 ml of the same buffer. The enzyme was eluted with a linear gradient of 0 to 0.4 M NaCl in 25 mM piperazine buffer with a counterion of piperazine chloride (pH 5.5) at a flow rate of 1 ml/min, and 1-ml fractions were collected. The fractions that exhibited enzyme activity were pooled and then concentrated to 2.8 ml with Centriprep 10. The 2.8-ml of concentrate was applied to a Butyl Toyopearl (Tosoh Co. Ltd.) column (1.6 by 6.5 cm) equilibrated with 25 mM citrate buffer (pH 5) containing 0.8 M $(NH_4)_2SO_4$. After the column was washed with 50 ml of the same equilibration buffer, proteins were eluted with a linear gradient of 0.8 to 0 M $(NH_4)_2SO_4$ in 25 mM citrate buffer (pH 5) at an elution rate of 1 ml/min, and 1-ml fractions were collected. Fractions corresponding to the main peak that exhibited enzyme activity were collected and divided into those corresponding to the left half of the peak and those corresponding to the right half of the peak. Each of the pooled proteins was dialyzed against 25 mM citrate buffer (pH 5) and concentrated to 2.8 ml with Centriprep 10. The dialyzed proteins were preserved at 4°C before being used in enzyme characterization.

Enzyme assay. Twenty-one types of dyes that were used in a previous study (18) and model compounds of RB5, AQ-1, AQ-2, and AQ-2' were used in the assay for purified enzyme activity.

We measured DyP activity in the supernatant of the culture broth by adding 1 ml of 25 mM citrate buffer (pH 3) to 2 ml of the supernatant to adjust its pH to 3.2 and then adding 119 μM RB5.

TABLE 1. Purification of peroxidase from supernatant of *G. candidum* Dec 1 culture

Purification step	Protein (mg)	Total activity (U)	Sp act (U/mg)	Activity yield (%)	Purification (fold)
Supernatant	88	24[a]	0.28[a]		1
Ultrafiltration (YM 10)	28	4.2×10^2	15	100[a]	55
Super Q chromatography	7.8	3.0×10^2	34	71	140
Butyl Toyopearl chromatography	1.5	85	57	20	210

[a] Total and specific activities were underestimated, presumably because of the presence of inhibitors in the supernatant; thus, the activity yield was calculated based on the value after YM 10 treatment.

For the enzyme assay of the samples obtained from the ultrafiltration and Super Q purification steps, crude enzyme solution (100 to 300 ng/ml) was incubated in 3 ml of 25 mM citrate buffer (pH 3.2) containing 119 μM RB5. Purified peroxidase (1.9 nM) eluted by Butyl Toyopearl chromatography was mixed with 3 ml of 25 mM citrate buffer containing each dye or model compound of RB5. DyP activity was measured with a spectrophotometer (UV-240; Shimadzu, Kyoto, Japan) at the maximum absorption wavelength of each dye and model compound at optimal pH. Measurement of DyP activity was initiated by the addition of 0.2 to 0.4 mM H_2O_2 at 30°C except for the assay of optimal temperature for decolorization. One unit of enzyme activity was defined as the amount of enzyme required for the decolorization of 1 μmol of RB5 or AQ-2' per min in the reaction mixtures. The value used was an average of three experiments, and the error was ±5%.

To assay the RB5-decolorizing activity of HRP, 1.7 nM HRP (with a molecular mass of 40 kDa) was used at an optimal pH of 4.0.

The 2,6-dimethoxyphenol oxidation activity of DyP was measured by the increase in absorbance at 470 nm (A_{470}) of the reaction mixture containing 25 mM citrate buffer (pH 4.5), 2.8 nM purified DyP, 0.2 mM 2,6-dimethoxyphenol, and 0.2 mM H_2O_2. The oxidation of guaiacol was measured in the same manner as that of 2,6-dimethoxyphenol except for the addition of 1 mM guaiacol instead of 2,6-dimethoxyphenol and measurement at A_{465}.

The RB5-decolorizing activity of purified DyP was measured at different temperatures to determine the optimal temperature. To compare the thermostabilities of DyP and HRP, DyP and HRP solutions in 25 mM citrate buffer were incubated at 40, 50, and 60°C and the activities of periodically sampled DyP and HRP were measured at 30°C.

Protein concentration. Protein concentration was measured by the Bradford method (5) with bovine gamma globulin (Bio-Rad) as the standard.

Determination of molecular mass and isoelectric point. The apparent molecular mass of purified DyP was estimated by gel filtration chromatography on a Sephacryl S-200 column (3.1 by 95 cm) eluted in 25 mM citrate buffer (pH 5). The standard proteins (Bio-Rad) used were thyroglobulin (670 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), gamma globulin (158 kDa), and vitamin B_{12} (1.35 kDa).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (type AE-6440; ATTO Co. Ltd., Tokyo, Japan) was performed in a 10% polyacrylamide gel. Reduced α-2-macroglobulin (170 kDa), phosphorylase *b* (97.4 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), and trypsin inhibitor (20.1 kDa) were used as standard-molecular-mass proteins for electrophoresis (Combithek; Boehringer Mannheim Yamanouchi Co. Ltd., Tokyo, Japan).

The isoelectric point (pI) of DyP was determined by isoelectric point electrophoresis (Multiphor II 2-D; Pharmacia) with a low-pI calibration kit (pH 2.5 to 6.5) (Pharmacia) as a standard pI marker.

Assay for hemoprotein. Purified DyP (7 μM) in 25 mM citrate buffer (pH 5) was scanned at 700 to 300 nm to identify the Soret band, and then 25 μM H_2O_2 was added to oxidize DyP and to allow observation of the shift of the Soret band. A small amount of $Na_2S_2O_4$ was added to the oxidized DyP to obtain the reduced form (44). The pyridine hemochrome content per mole of DyP was estimated by using the molar extinction coefficient of pyridine hemochrome (33×10^3 M^{-1} cm^{-1} at 556 nm), as described for cytochrome b_2 (1).

Sugar analysis. DyP (50 μg) was dried at 100°C and then hydrolyzed in 100 μl of 2.5 M trifluoroacetic acid at 100°C for 6 h. Then it was dried to remove the trifluoroacetic acid and coupled with 2-aminopyridine, a fluorescent compound, as described by Hase et al. (14). The reactant was adjusted to pH 9 by adding NH_4OH, and a two-phase separation was conducted seven times with chloroform to remove excess 2-aminopyridine. The hydrolyzed sugars were eluted with 0.25 M citrate buffer containing 1% acetonitrile (pH 4) by high-pressure liquid chromatography with ODS-120T (Tosoh Co. Ltd.) at a rate of 1 ml/min as described previously (15, 44). Glucosamine (Glc), mannose (Man), fucose (Fuc), *N*-acetylmannosamine (ManNAc), *N*-acetylglucosamine (GlcNAc), and *N*-acetylgalactosamine (GalNAc) were used as standard sugars.

Comparison of the dye-decolorizing activity of DyP and HRP. The RB5-decolorizing activities of DyP and HRP were measured at optimal pH values of 3.2 and 4.0, respectively. To obtain pseudo-first-order kinetic constants, the necessary concentrations of enzymes were used so that the decolorization rates of the dyes were not limited by substrate concentration. The concentrations of DyP and HRP were determined to be 1.3 and 1.7 nM, respectively. The concentration of RB5 was varied from 24 to 119 μM. The concentration of H_2O_2 varied from 10 to 20 μM to avoid inhibiting the enzyme activity. The $K_{m(obs)}$ of RB5 was estimated at 0.2 mM H_2O_2 from a plot of enzyme activity against RB5 concentration. $K_{m(obs)}$ of H_2O_2 was estimated at a fixed RB5 concentration of 119 μM from the relationship between enzyme activity and H_2O_2 concentration. $K_{cat(obs)}$ of DyP was estimated from V_{max} obtained from the reciprocal plot between DyP activity and RB5 concentration (36). Each value was obtained in triplicate, and the average value was used. The error was ±10%.

Second-order kinetics. AQ-2', a simplified form of RB5, was used as a substrate mainly because the by-products derived from RB5 degradation inhibited DyP activity, as described previously (18), and the mechanism of DyP activity can be clarified easily by using a simplified substrate. The concentration of DyP was fixed at 2.5 nM, and that of AQ-2' varied from 60 to 150 μM. H_2O_2 was used in the range of 20 to 80 μM, because the inhibitory effect of H_2O_2 on DyP activity was observed at low concentrations when AQ-2' was used. DyP activity for AQ-2' was measured at the optimal pH of 3.2. $K_{m(AQ\text{-}2')}$ was obtained from the Lineweaver-Burk plot (first plot) of AQ-2' concentration against enzyme activity. V_{max}, $K_{m(H_2O_2)}$, and K_{cat} (turnover rate) were determined from the double-reciprocal plot (second plot) of $V_{max(app)}$ against H_2O_2 concentration, as described previously (17, 36). The average value of triplicate data was employed, and the error was ±10%.

RESULTS

Enzyme purification. The total enzyme activity of the intact supernatant was 24 U, as shown in Table 1. The activity increased to 4.2×10^2 U after ultrafiltration with a YM 10 membrane because inhibitory substances with molecular masses of less than 10 kDa were removed (20). One peak that eluted at 0.13 M on the Super Q 650M column NaCl gradient possessed DyP activity. The elution pattern of the Butyl Toyopearl column, in which fractions that corresponded to the main peak exhibiting DyP activity were eluted at 0.57 M $(NH_4)_2SO_4$, is shown in Fig. 1. Fractions 86 to 99, corresponding to the left half of the main peak, were pooled and called purified DyP. The other fractions (100 to 146), which also possessed enzyme

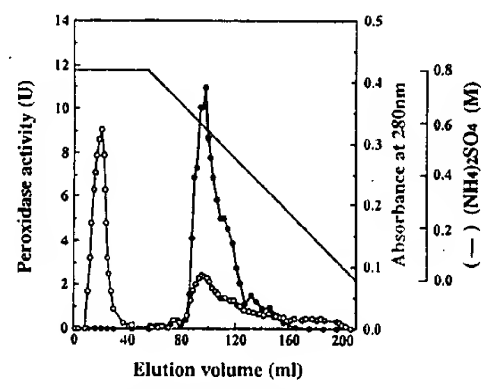

FIG. 1. Butyl Toyopearl chromatography of a peroxidase produced by *G. candidum* Dec 1. Symbols: ●, peroxidase activity; ○, absorbance at 280 nm.

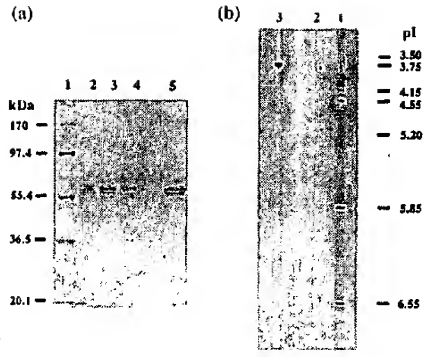

FIG. 2. (a) SDS-polyacrylamide gel electrophoresis of DyP after each purification step. Lanes: 1, standard molecular mass markers; 2, sample after YM 10 ultrafiltration (amount of protein loaded, 10 μg); 3, sample after Super Q chromatography (amount of protein loaded, 10 μg); 4, purified DyP after Butyl Toyopearl chromatography (amount of protein loaded, 6 μg); 5, mixed DyPs after Butyl Toyopearl chromatography. (b) Isoelectric focusing of purified DyP and mixed DyPs obtained from Butyl Toyopearl chromatography. Lanes: 1, mixed DyPs after Butyl Toyopearl chromatography (amount of protein loaded, 5 μg); 2, purified DyP after Butyl Toyopearl chromatography (amount of protein loaded, 10 μg); 3, standard pI marker.

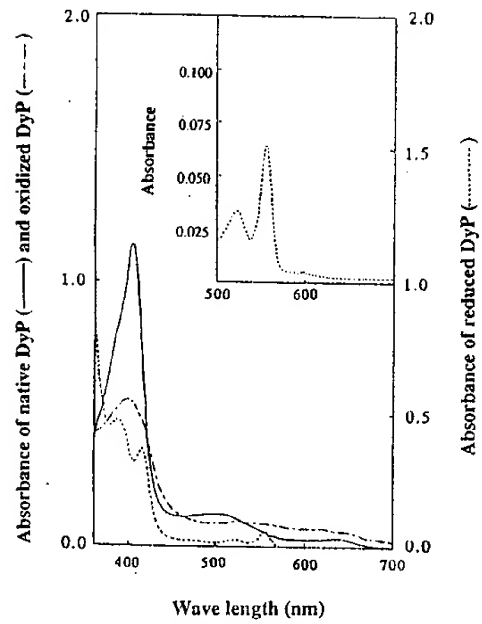

FIG. 3. Spectral characteristics of purified DyP, DyP oxidized by H_2O_2, and DyP reduced by dithionite. The inset shows the peak at 556 nm indicative of reduced DyP in the form of a heme-pyridine complex.

activity, were pooled and called mixed DyPs, because they are presumed to be composed of several kinds of DyP isozymes.

Purified DyP has a specific activity of 57 U/mg of protein and a recovery ratio of 20% (Table 1). The molecular mass of purified DyP as estimated by SDS-polyacrylamide gel electrophoresis was 60 kDa (Fig. 2a), revealing DyP to be a monomer, because the apparent molecular mass determined by gel filtration with Sephacryl S-200 was 55 kDa. The isoelectric point of DyP was 3.8 (Fig. 2b).

The mixed-DyP fraction consisted of mixed proteins whose molecular masses ranged from 55 to 60 kDa (Fig. 2a, lane 5) with a pI of 3.8 (Fig. 2b), and it had a specific activity of 57 U/mg of protein, which was about half the total activity (110 U) of DyP, corresponding to the main peak on Butyl Toyopearl chromatography.

Spectral characteristics. The spectral characteristics of purified DyP are shown in Fig. 3. A large Soret band was observed at 406 nm, together with two small peaks at 510 and 640 nm. The estimated molar extinction coefficient at 406 nm was $0.9 \times 10^5\ M^{-1}\ cm^{-1}$, similar to those of other peroxidases (25, 35, 40, 48). The A_{406}/A_{280} (RZ) value, which reflects the purity and spectral characteristics of DyP, was 1.6 in 25 mM citrate buffer (pH 5). When H_2O_2 was added to the purified DyP, the peaks at 406 and 510 nm were shifted to the peak at 400 nm, with a molar extinction coefficient of $4 \times 10^4\ M^{-1}\ cm^{-1}$, and the peak at 530 nm, with a coefficient of $6.7 \times 10^3\ M^{-1}\ cm^{-1}$, respectively. When DyP that was oxidized by H_2O_2 was reduced by $Na_2S_2O_4$, a peak at 556 nm, which corresponded to a heme-pyridine complex, appeared. From these results we concluded that DyP has a protoheme as its prosthetic group. The heme content per mole of DyP was estimated as 0.6, indicating that DyP retained a single heme.

Substrate specificity. Purified DyP was used to decolorize the 21 dyes (Table 2) which were decolorized by Dec 1 (18). DyP decolorized seven dyes containing azo and anthraquinone groups and three model compounds of RB5. Higher decolorizing activity was observed for anthraquinone dyes than for azo dyes. Phenolic compounds 2,6-dimethoxyphenol and guaiacol, which are substrates of MnP, were degraded by DyP, but veratryl alcohol, a well-known substrate of LiP, was not (data not shown). The oxidation of 2,6-dimethoxyphenol and guaiacol occurred without the addition of Mn^{2+} at optimal pH values of 4.5 and 4.0, respectively, and no enhancement of the DyP activity by the addition of Mn^{2+} was observed. These results confirmed that DyP had a wide degradation spectrum and that its substrate specificity differs from those of LiP and MnP.

Sugar analysis. Fifty micrograms of DyP contained 8.7 μg of sugar, which was composed of GlcNAc (38 mol per mol of DyP) and Man (26 mol per mol of DyP). This result indicates that DyP is a glycoprotein containing 17% (wt/wt) sugar.

Optimal temperature for and thermostability of DyP activity. The effect of temperature on DyP activity is shown in Fig. 4. DyP activity was optimal at 30°C, and relatively high activity was maintained in the range of 15 to 35°C (Fig. 4a). The thermostabilities of DyP and HRP activities were measured at 30°C after treatment at various temperatures (Fig. 4b). DyP activity was restored at 30°C even after treatment at 40 and 50°C for 11 h. When DyP and HRP were heated at 60°C for 3 h, 35 and 90% of their initial activities respectively, were lost. These results suggest that DyP is more thermostable than HRP. Additionally, when DyP was incubated at 30 and 40°C for 14 days, the inactivation rates were 37 and 59%, respectively.

H_2O_2 inhibition of DyP activity. When RB5 and AQ-2′ were used as substrates, the inhibitory effect of H_2O_2 on DyP activity was observed (Fig. 5). DyP activity for RB5 was inhibited when the H_2O_2 concentration exceeded 0.2 mM at a fixed DyP concentration of 0.6 nM. At 2.8 nM of DyP, its activity for AQ-2′ decreased sharply when the H_2O_2 concentration exceeded 0.1 mM, and it was lower than that for RB5. The degree of inhibition by H_2O_2 of DyP activity differed significantly depending on the substrate used.

Comparison of decolorizing activities of DyP and HRP. Pseudo-first-order kinetics was applied to compare the rates of decolorization of RB5 by DyP and HRP (Table 3). DyP had

TABLE 2. Enzymatic activity of purified DyP on various dyes

Color index of dyes	Chromophore	ε at λ_{max}[a] (liters/mM/cm)	Optimal pH	Initial concn (μM)	Decolorizing rate (μM/min)
RB5	AQ[b]	7.3	3.2	119	24
RB19	AQ	10	3.2	116	22
RB114	AQ	8.4	4.0	151	12
AQ-1	AQ	9.0	3.2	113	10
AQ-2	AQ	8.3	3.0	141	55
AQ-2'[d]	AQ	7.3	3.2	148	20
Reactive Black 5	AZ[c]	37	3.2	33	1.1×10^{-1}
Reactive Red 33	AZ	23	3.2	60	4.7×10^{-1}
Reactive Yellow 2	AZ	8.1	3.2	125	6.2×10^{-1}
RB182	AZ	7.3	4.0	123	21

[a] Molar extinction coefficient at maximum absorption wavelength (λ_{max}) of each dye.
[b] AQ, anthraquinone.
[c] AZ, azo.
[d] Decolorization test of AQ-2' by Dec 1 was not conducted in the previous study (18).

slightly lower $K_{m(RB5)}$ and $K_{m(H_2O_2)}$ values than HRP, and the $K_{cat(obs)}$ of 260 s^{-1} for DyP was 1.8 times higher than that for HRP (140 s^{-1}). When RB5 solution was scanned after decolorization by both enzymes, the spectral patterns were considerably different at wavelengths below 450 nm, reflecting the difference in the by-products produced from RB5 by the two peroxidases.

Second-order kinetics of DyP activity. In the DyP turnover involving the two substrates AQ-2' and H_2O_2, the apparent $K_{m(AQ-2')}$ was initially obtained from the Lineweaver-Burk plot of AQ-2' concentration and DyP activity. Then the V_{max}, $K_{m(H_2O_2)}$, and apparent K_{cat} were determined from the reciprocal plot of $V_{max(app)}$ and H_2O_2 concentration. The estimated apparent kinetic constants are shown in Table 4. $K_{cat}/K_{m(AQ-2')}$ and $K_{cat}/K_{m(H_2O_2)}$, which are physicochemical constants of the substrates, were 3.2×10^6 and 7.6×10^6 M^{-1} s^{-1}, respectively. The apparent K_{cat} was similar to the $K_{cat(obs)}$ when RB5 was used as the substrate, indicating a similar turnover rate of a more simply structured substrate, AQ-2', by DyP.

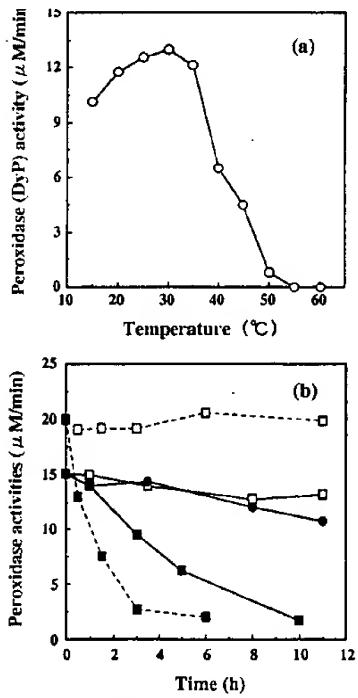

FIG. 4. (a) Optimal decolorization temperature of DyP. (b) Thermostability of DyP and HRP; enzyme activity at 30°C after treatment with DyP at 40°C (□——□), DyP at 50°C (●——●), DyP at 60°C (■——■), HRP at 40°C (□----□), and HRP at 60°C (■----■).

DISCUSSION

A peroxidase that transformed anilines was purified from the culture broth of a strain of *G. candidum* (4). DyP was produced under aerobic conditions as a secondary metabolite in the stationary phase and reached its maximum level at day 6 (18). Since DyP activity is maintained as long as sugars exist in basal III mineral medium (24), a medium frequently used in LiP production, DyP of strain Dec 1 is considered to be a constitutive enzyme. LiP of *Phanerochaete chrysosporium* was produced under limited-nitrogen conditions (8, 9, 42). It required inducers, such as veratryl alcohol and veratryl acid, for production, and oxygen-enriched aeration (9) and low shear stress (21, 24) were essential to obtain higher activity. However, the production of peroxidase by Dec 1 is more efficient and convenient than that by *P. chrysosporium*, because DyP is constitutively produced during shaking cultivation (18) or in a stirred tank reactor (19) without a marked decrease in activity.

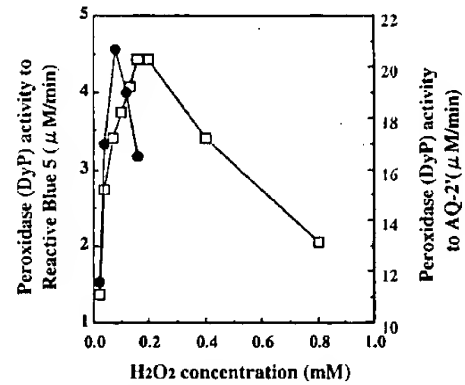

FIG. 5. H_2O_2 inhibition of DyP activity when RB5 and its simplified model compound, AQ-2', were used as substrates. Symbols: □, degradation activity of 0.6 nM DyP for RB5; ●, degradation activity of 2.8 nM DyP for AQ-2'.

TABLE 3. Kinetic parameters for activities of DyP and HRP[a]

Parameter	Unit(s)	Value	
		DyP	HRP
$K_{m(RB5)}$	μM	54	58
$K_{m(H_2O_2)}$	μM	26	36
$K_{cat(obs)}$	s^{-1}	260	140
$K_{cat}/K_{m(RB5)}$	$M^{-1}\ s^{-1}$	4.8×10^6	2.4×10^6
$K_{cat}/K_{m(H_2O_2)}$	$M^{-1}\ s^{-1}$	1.0×10^7	3.8×10^6

[a] RB5 decolorization by DyP and HRP was determined by pseudo-first-order kinetics.

Electrophoretic analysis of DyP gave a pI of 3.8. Although molecular mass values obtained by SDS-polyacrylamide gel electrophoresis and by gel filtration differed slightly, i.e., 60 and 55 kDa, respectively, DyP is considered to be a monomer. The difference in molecular mass may be due to the gel filtration value being underestimated, presumably because DyP is not spherical or the sugar portion of DyP is interacting with the gel matrix. The mass of DyP was considerably larger than those reported previously (10, 27, 45, 48, 51), which ranged from 40 to 44 kDa. Kang et al. (17) reported a 140-kDa peroxidase with two subunits of 72 kDa. In addition to DyP, Dec 1 produced mixed DyPs that were eluted by Butyl Toyopearl chromatography and had the same pI as purified DyP. Since there are small differences in molecular mass among them, mixed DyPs are assumed to consist of proteins with amino acid sequences identical to that of purified DyP but with different sugar contents.

DyP degraded 7 of the 18 dyes that were decolorized by Dec 1, indicating that other enzymes contributed to the broad decolorizing spectrum of Dec 1. DyP showed a higher decolorizing rate for anthraquinone dyes than for azo dyes (Table 2). However, high decolorizing activity was obtained for the azo dye RB182. This is presumably because this dye contains Cu in its structure. DyP degraded phenolic compounds, such as 2,6-dimethoxyphenol and guaiacol, as well as a variety of dyes, while it did not degrade nonphenolic veratryl alcohol. Considering its substrate specificity and molecular mass, purified DyP is thought to be a novel decolorizing peroxidase, distinct from LiP, MnP, HRP, and other peroxidases reported previously. The enzymes that were involved in lignin degradation also degraded various aromatic compounds, lignin model compounds (33), and synthesized dyes (6–8, 11, 13, 22, 30, 43, 47). The wide degradation spectra may depend on the involvement of an active oxygen species and/or radicals (hydroxyl, etc.) in the degradation (2, 33). The broad degradation spectrum of DyP could also be due to the presence of an active oxygen and/or radicals initially produced by DyP.

The spectral characteristics of DyP are similar to those of typical peroxidases. The Soret band, which is the representative absorption peak of peroxidase (16, 34, 48), was observed at 406 nm for native DyP. When H_2O_2 was added to native DyP, the Soret band shifted to 400 nm, presumably due to the formation of compound I of DyP, which resembles LiP L3 from *Phlebia radiata* (25). Since the DyP reduced by dithionite gave a new peak at 556 nm, assigned to a pyridine hemochrome, we concluded that DyP had a protoheme as its prosthetic group, similar to HRP and LiP (40).

H_2O_2 inhibition of DyP activity was observed (Fig. 5). The inhibition of HRP or LiP activity by excess H_2O_2 is well known (26, 49). In the turnover cycles of these peroxidases, they are first oxidized by two electrons from H_2O_2 to compound I. Then, one electron is removed by a substrate, changing compound I to compound II. Compound II is further reduced to a resting enzyme by another electron from a substrate. However, in the presence of excess H_2O_2, compound II that cannot be converted to a resting enzyme is changed to compound III, an inactivated state, which decreases peroxidase activity (26, 46, 49). In the case of DyP, conversion to compound I (Fig. 3) was suggested by shifts of the peak at 406 nm to 410 nm and of the peak at 510 nm to 530 nm, which were similar to those in previous reports (25, 37). Details of H_2O_2 inhibition of DyP activity were not elucidated, but a mechanism similar to that described above may be involved. Furthermore, the extent of inhibition is dependent on the substrate; the inhibition was observed at a lower H_2O_2 concentration for the simplified substrate AQ-2′ even if the DyP concentration was higher than the RB5 concentration.

Sugar analysis revealed that DyP contained 17% (wt/wt) sugar (GlcNAc and Man) in its structure. The GlcNAc and Man contents per mole of DyP were 34 and 23 mol, respectively. HRP is composed of 16 to 18% sugar, although the sugar content differs depending on the isozyme (35, 40, 51). The sugar content of LiP varied widely, from 17% at a pI of 3.8 to 39% at a pI of 4.2 (12). The nature of the sugar linkage in DyP is not yet clear, but LiP retained both N-linked sugar chains and O-linked monosaccharides (38), while HRP containing 18% sugar had only asparagine N-linked types at eight sites per mole (51). The N-terminal amino acid of DyP was not detected by the Edman procedure, indicating that it may be blocked by sugars (17, 41). Cloning of *dyp* is necessary to clarify the structure of DyP.

DyP activity was maximal at 30°C, and it was maintained at a high level at temperatures ranging from 20 to 35°C (Fig. 4a). However, according to the present experimental procedure, residual DyP activity was stable even after incubation at 50°C for 11 h and DyP revealed higher thermostability than HRP, in particular, at 60°C (Fig. 4b). This is presumably due to its larger molecular mass with a sugar attached and its high thermal reversibility, because a partial recovery or a reversible thermal inactivation of HRP and peroxidase-M2 activities was also observed (29, 34, 48).

The decolorizing activities of DyP and HRP for RB5 were compared by analysis of pseudo-first-order kinetics (Table 3). Although the K_m values for RB5 were almost the same for the two peroxidases, the K_m of DyP for H_2O_2 was lower than that of HRP, indicating a higher affinity of DyP to H_2O_2. $K_{cat(obs)}$, which represents DyP activity, was 1.8 times that of HRP. High decolorizing activity of DyP for a synthetic dye may have resulted from its higher redox potential or its affinity for a substrate. By second-order kinetic analysis of DyP activity with AQ-2′ and H_2O_2, the apparent kinetic parameters, $K_{m(AQ\text{-}2')}$, $K_{m(H_2O_2)}$, and K_{cat}, were estimated (Table 4). Since the values of K_{cat} of LiP for veratryl alcohol and of MnP for Mn(II) were reported to range from 30 to 160 s^{-1} (12, 27, 30), a higher turnover rate of DyP on dyes is suggested by the results of this experiment. The apparent K_{cat} of AQ-2′ was similar to that of RB5. This suggests the usefulness of using AQ-2′ to elucidate

TABLE 4. Second-order kinetic constants of DyP with H_2O_2 and AQ-2′ as substrates

Parameter	Unit(s)	Value
$K_{m(AQ\text{-}2')}$	μM	84
$K_{m(H_2O_2)}$	μM	36
K_{cat}	s^{-1}	270
$K_{cat}/K_{m(AQ\text{-}2')}$	$M^{-1}\ s^{-1}$	3.2×10^6
$K_{cat}/K_{m(H_2O_2)}$	$M^{-1}\ s^{-1}$	7.6×10^6

the mechanism of degradation of a dye by DyP and to compare it with those of the other peroxidases (17, 23, 25, 50), because AQ-2′ is a simplified structure of RB5.

In our previous paper, we showed that *G. candidum* Dec 1 decolorized 18 kinds of dyes and three model compounds. Purified DyP showed high activity for anthraquinone dyes and decolorized seven dyes. Mixed DyPs containing several isozymes decolorized 15 kinds of dyes (data not shown). However, purification of each isozyme is sometimes difficult due to the structural similarities. Therefore, we plan to clone the DyP gene (*dyp*) and use this DNA sequence to isolate genes which encode isozymes. We expect these isozymes to be sufficient to explain the broad decolorization spectrum of this strain.

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